Dust and bullets: stable isotopes and GPS tracking disentangle lead sources for a large avian scavenger

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Abstract

Lead intoxication is an important threat to human health and a large number of wildlife species. Animals are exposed to several sources of lead highlighting hunting ammunition and lead that is bioavailable in topsoil. Disentangling the role of each in lead exposure is an important conservation issue, particularly for species potentially affected by lead poisoning, such as vultures. The identification of lead sources in vultures and other species has been classically addressed by means of stable-isotope comparisons, but the extremely varied isotope signatures found in ammunition hinders this identification when it overlaps with topsoil signatures. In addition, assumptions related to the exposure of individual vultures to lead sources have been made without knowledge of the actual feeding grounds exploited by the birds. Here, we combine lead concentration analysis in blood, novel stable isotope approaches to assign the origin of the lead and GPS tracking data to investigate the main foraging grounds of two Iberian griffon vulture populations (N=58) whose foraging ranges differ in terms of topsoil lead concentration and intensity of big game hunting activity. We found that the lead signature in vultures was closer to topsoil than to ammunition, but this similarity decreased significantly in the area with higher big game hunting activity. In addition, attending to the individual home ranges of the tracked birds, models accounting for the intensity of hunting activity better explained the higher blood lead concentration in vultures than topsoil exposure. In spite of that, our finding also show that lead exposure from topsoil is more important than previously thought.

Key words:

Lead, ammunition, ecotoxicology, GPS, vultures, stable isotopes

1. Introduction

Lead is a heavy metal whose toxic effects in humans have been known for millennia (Papanikolau et al., 2005). Its consequences in wildlife, however, were not described until the 19th century (Calvert 1876). Since then, direct mortality due to lead toxicity has been frequently reported for many avian species (Pain et al., 2019). There are, nonetheless, more subtle and bearly detectable sub-lethal effects that often go unnoticed, such as alterations in behavior, morphology, and breeding success or physiological functions (Espín et al., 2015; Golden et al., 2016; Vallverdú-Coll et al., 2016). Consequently, the study of the impact of lead pollution on wildlife has become an extremely active field in conservation of threatened populations (Pain et al., 2019).

Vultures are one of the bird groups most sensitive to lead intoxication to the extent that it has been noted as a significant conservation problem for many vulture species worldwide (Golden et al., 2016; Plaza & Lambertucci 2019), threatening entire populations and compromising the success of costly conservation programs (Finkelstein et al., 2012). The obligate scavenging habits of vultures make them very prone to ingesting ammunition from big game hunting remains (Mateo et al., 1997; García-Fernández et al., 2005; Krone 2018). Carcasses and remains of shot animals are frequently abandoned in nature (Hunt et al., 2006; Legagneux et al., 2014) and can contain up to hundreds of fragments of metallic lead that can be bioavailable for vultures because of the characteristic extremely acidic gastric fluid of these species (Hunt et al., 2006; Hunt et al., 2009; Knot et al., 2010).

Ammunition is not the only source of lead that could affect vultures. Alternative sources of lead such as paint, contaminated water or soils have also been described as possible causes of intoxication in wildlife (Katzner et al 2018). Some of them, such as lead-based paint, are of little relevance to scavengers because of their low exposure occurrence (Finkelstein et al., 2102). On the contrary, lead in soil is naturally widespread, and mining activities have led to its bioavailability to wildlife. This is relevant because wild and domestic ungulates, whose carcasses are the main food source for vultures, accumulate lead from the soil in their tissues triggering potential trophic transfer processes affecting higher trophic levels (García-Fernández, 2014; Mateo-Tomás et al., 2016; Naidoo et al., 2017).

Starting from this scenario, it is crucial to identify the role that ammunition and topsoil lead play in vulture intoxication, not only to counteract resistance to global regulations on lead hunting ammunition (Cromie et al., 2014), but also to rule out possible underestimates of the risk posed by topsoil lead. Thus far, the most direct approaches have made use of stable isotope signatures (Church et al 2006; Mateo-Tomás et al., 2016; Naidoo et al., 2017). In addition, the application of stable isotope mixing models goes one step further, allowing a detailed assessment of the contribution of potential lead sources (Longman et al., 2018). This approach alone, however, is incomplete. It is well known that large avian scavengers perform huge long-distance movements (Alarcón & Lambertucci 2018), which makes it difficult to determine where the individuals may have been exposed to lead in topsoil and/or game carcasses (Binkowski et al., 2016). In addition, from a population point of view, individual foraging decisions are highly variable (Alarcón & Lambertucci 2018), which implies the possibility that different birds in the same breeding area could be unequally exposed to different lead sources. Recent studies have tried to deal with this but have been based on direct observations (Church et al., 2006; Mateo-Tomás et al., 2016; Naidoo et al., 2017), which can introduce important biases when the home ranges are very large or include poorly accessible areas.

Here, taking advantage of GPS tracking of 58 griffon vultures of two Spanish populations differently exposed to topsoil and ammunition, we aim to identify the contribution of topsoil and ammunition sources to lead concentrations in the blood of the tracked birds. Spain is an excellent place to address this issue because it holds 90% of the European population and shows a high prevalence of abnormal blood lead levels (García-Fernández et al., 2005;Mateo-Tomás et al. 2016; Descalzo and Mateo 2018). Moreover, Spanish vultures are exposed to both target lead sources. Whereas elevated lead exposure has been reported in wild ungulates, as well as in livestock, because of topsoil contamination in some Spanish regions (Reglero et al., 2009, Taggart et al., 2011, Pareja-Carrera et al., 2014), the populations of these game species are recovering across most of the country, with the number of animals hunted being one of the largest in Europe (Apollonio et al 2010). Our aim is to estimate for the first time, linkages between sources of lead in the environment and that found in griffon vultures and the spatial scale at which this species may be exposed to lead. We specifically predict that 1) blood lead in individual vultures derives from two different sources, ammunition and topsoil; 2) lead in the blood of vultures differs between populations based on the individual level of exposure to topsoil and ammunition; and 3) exposure to big game hunting is the major driver of high levels of blood lead concentration.

2. **Methods**

2.1. *Focus species and study area*

The European griffon vulture is a large body-sized (up to 12 kg) obligate scavenger. It is the most abundant European vulture (Margalida et al., 2010). The bulk (90%) of the European populations are concentrated in Spain (Margalida et al., 2010) where a 2018 census estimated 30.946 breeding pairs (del Moral and Molina 2018). They nest on cliffs and their main source of food is domestic and wild ungulates (Margalida et al., 2011). They feed over areas covering thousands of square kilometers (Arrondo et al., 2018) and thus rely on social information (Cortés-Avizanda et al 2014).

We captured and tagged 58 adult (more than seven years old) griffon vultures in two distant populations (hereafter “southern” and “northern”) of the Iberian Peninsula (see Arrondo et al 2019). Captures were done at baited sites by means of cannon-nets. Thirty birds were trapped in Sierra de Segura Cazorla y las Villas Natural Park, Southern Spain (Figure 1) in December 2014. The movements of these vultures extend mainly westwards to the Portuguese border (see Arrondo et al., 2018). This area is dominated by Mediterranean woodlands and “dehesas”, which are traditional silvopastoral landscapes where two of the main economic activities are traditional livestock (including free-ranging herds of sheep and pigs) and big game hunting (Acevedo et al., 2011). In addition, this area has hosted significant lead-mining operations for centuries (Reglero et al., 2009). The other 28 vultures were captured in Bardenas Reales Natural Park, Northern Spain (Figure 1) in December 2015. In this area, griffon vultures are mainly concentrated around Ebro Valley, a relatively flat area mainly characterized by irrigated crops and intensive livestock farms and surrounded by mountain ranges with Mediterranean woodlands and pastures (Lecina et al., 2005; Martín-Queller et al., 2010). Big game hunting is common but less intense than in the southern area (Acevedo et al., 2014). In addition, there is no history of lead mining activity, but natural lead is present at high concentrations in mountain topsoil (Locutura et al., 2012). Additionally, griffon vultures from this population also travel long distances to Southwestern Iberia, where they share some foraging zones with vultures from the southern population (Arrondo et al., 2018, 2020 and Figure 1).

Trapping and handling were carried out with the proper permits and bioethical authorizations. During handling, safety protocols were followed to avoid stressing the animals. Until the moment of the tagging, the individuals were isolated and safe. The tagging was always done by at least two people and never lasted more than twenty minutes.

All the individuals were tagged with 90 g GPS/GPRS-GSM backpack devices from E-OBS Digital Telemetry (https://www.e-obs.de/http://www.e-obs.de). Devices were equipped as backpacks using a Y type harness made of Teflon following the procedures described in Kenward (2000). The devices were programmed to record variable numbers of locations depending on weather conditions and the power level of the batteries. During spring and summer, devices recorded one fix every 5 minutes if the battery was full, every 20 minutes if the battery was half-full and every 30 minutes if the battery was close to empty. In autumn and winter, the devices recorded every 10 minutes if the battery was full, every 30 minutes if the battery was half-full and every 60 minutes if the battery was close to empty. Throughout the year, if the batteries were discharged below the safety level, the device would only record one fix per day. We compiled movement data for all birds since the capture until December 2018 unless the animal died or the device failed (Table S.1.).

2.2. *Lead analysis and isotopic determination*

We took blood samples by brachial puncture from all of the individuals. Whole blood without anticoagulant was stored at -20 ºC until the analyses of blood lead concentration and isotope composition. Blood samples were also used to determine the sex of the birds by molecular procedures (Wink et al., 1998).

Blood samples (0.4-1.0 g) were digested with 3 ml of HNO_3 (69% Analytical Grade), 1ml of H_2O_2 (30% v/v Suprapur) and 4 ml of H_2O (Milli-Q grade) with a microwave oven (Ethos E, Milestone) (Reglero et al., 2009). Lead concentrations and the proportion of the stable isotopes ^{206}Pb, ^{207}Pb, and ^{208}Pb were measured in the digested solutions by inductively coupled plasma quadrupole mass spectrometry (ICP-MS) following Martínez-Haro et al., (2011).

Stable lead isotope composition was also analyzed in the topsoil of mining sites of Sierra Madrona-Valle de Alcudia (Table S.2). Here, elevated lead concentrations have been detected in soil (average values of different sites: 7.78-8897 µg/g; Reglero et al., 2008; Rodríguez-Estival et al., 2014) and in wild ungulates (red deer and wild boar muscle showed geometric means with 95%CI of 0.483 (0.32-0.73) and 2.63 (1.13-6.15) µg/g) and the livestock (sheep liver showed 6.16 (4.12-9.23) µg/g; Reglero et al., 2009, Taggart et al., 2011, Pareja-Carrera et al., 2014). Additionally, vultures from both populations usually forage in this area (Figure 1). The isotope ratios in topsoil lead of this region are very similar to that found in the northern study area (Monna et al 2004). Soil samples (≈ 100 g) were taken at a depth of 0-5 cm using a shovel and stored in independent ziplock polyethylene bags. Soil samples were oven-dried, disaggregated in a mortar and sieved through a 250 µm-aperture nylon mesh before being acid-digested (0.2 g) as described above.

We also determined the isotopic composition of the most frequently used lead-based bullets in Spain. For this purpose, we obtained 17 bullets and 3 cartridges of 6 commercial brands (Table S.2).

Blanks and a certified reference material of lobster hepatopancreas (TORT-2) with 0.39 µg/g of lead were processed in each batch of digestions. The limit of detection (LODs) of lead in blood was 0.32 µg/dl. We calculated blood lead concentration in µg/dl considering blood density at 1.06 g/ml to make our results more comparable with the available literature. The mean (± %RSD) lead recovery in the reference material TORT-2 was 94.7% (± 5.8%, n = 12). The precision expressed as %RSD was lower than 5.5% for lead concentration data (n=12).

Key operating conditions for isotope determination were quadrupole dwell time (10 ms for ^{206}Pb and ^{207}Pb and 5 ms for ^{208}Pb), number of scans per sample (800 sweeps), and dead time correction factor (35 ns). Both internal (203Tl/205Tl ratio) and external (NIST SRM 981, certified isotopic composition (mean ± 95%) of 24.144 ± 0.006% for ^{206}Pb, of 22.083 ± 0.003% for ^{207}Pb, and of 52.347 ± 0.009% for ^{208}Pb) standards were used for mass discrimination correction. All isotope ratios determined for SRM 981 during analysis were within an uncertainty <1% of the certified value (before a nominal rolling correction was applied to all data). For isotopic analysis, six replicates of each sample were run. Variability in isotopic data expressed as %RSD (n=6) was in all cases lower than 0.28%. Detailed values for each lead isotope ratio and type of sample are shown in Table S.3.

2.3. *Spatial variables*

We estimated the home ranges of GPS-tracked griffon vultures exclusively during the big game hunting period (October to March). Since the birds were captured in the middle of this period (in December, see above), we assume that the lead concentration levels recorded are representative of the lead exposure during whole hunting period. To ensure that core and foraging areas do not show a significant spatial variation during the study period, we assessed the stability of home ranges. According to Fieberg and Kochanny (2005), we used the Bhattacharyya's affinity (BA) index and the home range estimators overlap (HRE).

Before performing home range estimations, we standardized our data by resampling the dataset until we obtained for each individual a fix every 30 minutes. Home range and overlapping analyses were done by means of bivariate kernel functions using the adehabitatHR package (Calenge & Fortmann-Roe 2013) run in R version 3.5.1 (R development core team 2018). Fixed 95% and 50% kernel density contours were calculated to estimate the majority of the foraging areas, KDE 95%, and the core (intensive use) areas, KDE 50%. We used as a smoothing parameter the ad hoc method with a resolution of one ha (Margalida et al., 2016).

Potential topsoil and ammunition exposures were estimated by means of proxy variables. In the first case, and on the basis of the national geochemical atlas (resolution 1x1m) elaborated by the Spanish Geological and Mining Institute (Locutura et al., 2012), we calculated the median lead concentration (mg/kg) at the superficial ground inside the KDE 50 and KDE 95 areas of each individual. Exposure to ammunition was estimated in relation to hunting statistics. We defined the hunting intensity in KDE50 and KDE95 as the sum of wild boars (*Sus scrofa*) and red deer (*Cervus elaphus*) culled (https://www.mapa.gob.es/es/desarrollo-rural/estadisticas/) in a 10 x10 km cell covering all of peninsular Spain (https://www.miteco.gob.es/es/biodiversidad/temas/inventarios-nacionales/inventario-especies-terrestres/inventario-nacional-de-biodiversidad/bdn-ieet-atlas-vert-mamif.aspxhttps://www.miteco.gob.es/es/biodiversidad/temas/inventarios-nacionales/inventario-especies-terrestres/inventario-nacional-de-biodiversidad/bdn-ieet-atlas-vert-mamif.aspx).

Statistical details of both variables are described in Table S.4.

2.4. *Statistical analyses*

2.4.1. Lead sources in individual vultures

To infer the potential origin of the lead present in the blood of vultures, we applied stable isotopic Bayesian mixing models (MixSIAR, Stock et al., 2018) using three lead stable isotopic ratios, $^{206}Pb/^{207}Pb$, $^{207}Pb/^{208}Pb$, and $^{206}Pb/^{208}Pb$, in the blood of each GPS-tracked vultures. We avoided the use of ^{204}Pb isotope because of its low presence in the isotopic signature, which could introduce analytical biases in the calculations of isotope ratios in biological samples with low lead levels. MixSIAR Bayesian isotopic mixing models estimate the potential contribution of each isotopically distinct potential origin of lead (in our case topsoil and ammunition sources) in the diet of the consumer (in our case griffon vultures) based on the lead isotopic values of the consumer and its potential source. MixSIAR estimates probability density functions using Markov chain Monte Carlo methods, and each model was run with identical parameters. Model convergence was determined using Gelman-Rubin and Geweke diagnostic tests (Stock & Semmens, 2016; Stock et al., 2018). Bayesian mixing models have been developed to allow flexible model specification in a rigorous Bayesian statistical framework (Phillips et al., 2014). We did not use trophic enrichment factors between vulture's blood and sources of lead because no trophic enrichment factor occurs with lead as occurs with nitrogen (Longman et al 2018).

2.4.2. Factors associated with blood lead concentration in vultures

We related the blood lead concentration, transformed by logarithm in base 10, to the explanatory variables using General Linear Models (Gaussian error distribution and identity linkage). The explanatory variables selected were: a) median topsoil lead concentration at KDE 50; b) median topsoil lead concentration at KDE 95; c) big game hunting intensity at KDE 50; d) big game hunting intensity at KDE 95, e) area of KDE50, f) area of KDE95 and g) sex.

The two spatial scales analyzed (KDE50 and KDE95) were highly correlated in all variables (topsoil lead concentration: $t = 6.94$, $df = 58$, $p < 0.001$, $r = 0.67$; big game hunting intensity: $t = 50.90$, $df = 58$, $p < 0.001$, $r = 0.99$; area: $t = 9.82$, $df = 58$, $p < 0.001$, $r = 0.79$). In addition, topsoil lead concentration and big game hunting intensity were correlated at both scales KDE50 and KDE95 (KDE50: $t = 6.52$, $df = 58$, $p < 0.001$, $r = 0.65$; KDE95: $t = 21.02$, $df = 58$, $p < 0.001$, $r = 0.96$). All correlated variables were modeled independently.

Model selection was done by means of the Akaike's information criterion corrected for small sample size (AICc). Models with $\Delta AICc < 2$ were considered equivalents. We discarded models including uninformative parameters, i.e. parameters whose 85% confidence interval overlapped with 0 (Burnham and Anderson, 2002).

3. **Results**

Lead values above the background and toxic levels (>20 µg/dl and > 50 µg/dl, Pain et al. 2019) appeared in 93.3% and 78.6 % of individuals from the southern population and 66.7% and 28.6 % of individuals from the northern population, respectively (Table 1). Vultures from the southern population showed significantly higher mean lead concentrations than those from the northern population (mean ± SD respectively: 64.0 ± 29.9 vs. 40.1 ± 25.3 µg/dl; $t = -3.324$, $df = 54.718$, $p = 0.002$). Females tended to show higher frequencies of toxic (> 50 µg/dl) lead concentrations than males: 72.7% vs. 63.2% and 37.5% vs. 28.6% of the birds in southern and northern populations, respectively (Table 1).

3.1. *Stable isotopic results*

We found higher stable isotope ratios of $^{207}Pb/^{208}Pb$ and $^{206}Pb/^{208}Pb$ in vultures sampled in the southern compared to the northern area (Figure 2; T-Student Tests; $^{207}Pb/^{208}Pb$, t=2.21, p=0.03; $^{206}Pb/^{208}Pb$, t=2.26, p=0.02). In contrast, both populations showed similar stable isotope ratios of $^{206}Pb/^{207}Pb$ (Figure 2; t=2.21, p=0.03). In the case of lead sources, ammunition always showed higher stable isotope ratios of $^{207}Pb/^{208}Pb$, $^{206}Pb/^{208}Pb$ and $^{206}Pb/^{207}Pb$ than topsoil (Figure 2).

Lead source estimates derived from isotopic mixing models revealed that, for both populations the isotopic signature seems to be closer to topsoil tan ammunition (Figure 3; T-Student tests; topsoil vs. ammunition; northern population, t=-9.33, p<0.001; southern population, t=-4.96, p<0.001). However, the importance of ammunition was higher in the southern than in the northern population (Figure 3; southern vs. northern population; topsoil, t=7.38, p<0.001; ammunition, t=-7.36, p<0.001).

3.2. *Modeling blood lead concentration*

Overlap between years of utilization distribution areas was high (60 ± 25%, BA index), while both KDE50% and KDE95% showed high stability (HRE: index showed 42.41% ± 31.69% and 47.93% ± 32.60% (indiv= 46; indv-year=286)).

We obtained three AIC-equivalent models explaining blood lead concentrations in vultures (Table S5 and Table 2). Two models showed an effect of exposure to ammunition from big game hunting based on the KDE50 and KDE95 with an additive effect of sex. The third model selected included an effect of topsoil lead concentration at KDE50. In spite of the equivalence of the three models, the one that included big game hunting intensity at KDE50 and sex presented a weight of 44%, more than double the models that included big game hunting intensity at KDE595 and topsoil lead concentration whose weights were 20% and 17%, respectively. That is, those individuals, especially females whose core areas are in areas with high intensities of big game hunting, have higher levels of lead in blood (Table. S.5)

4. **Discussion**

Our results reveal that both topsoil and ammunition are important sources of lead found in the blood of griffon vultures, but their relative contribution is clearly asymmetric. Most of the vultures were exposed to background lead levels probably derived from both direct topsoil exposure (e.g. contaminated dust inhalation or ingestion) and a transfer between trophic levels. Toxic levels of lead is mainly explained, however, by the ingestion of hunting ammunition. Thus, our study, with the combination of GPS and isotopic signatures of blood lead analyses, is the first to provide a fine-tuned approach to disentangling how fine-scale foraging patterns determine individual variations in the contribution of different sources of lead.

4.1. *Sources of lead exposure in griffon vultures*

Our results showed that topsoil could has an important contribution to the lead found in vultures which could be explained by chronic exposure to this source compared to the exceptional exposure to ammunition. Topsoil lead is widely present in foraging areas of both northern and southern populations. The bulk of the vultures' diet is domestic and wild ungulates that are consistently exposed to lead from the topsoil, especially in mining areas (Reglero et al., 2009; Taggart et al., 2011; Pareja-Carrera et al., 2014). Apart from this, the remains of hunted wild ungulates in regions with topsoil lead would contain lead from both sources (topsoil and ammunition). It should also be noted, that the average concentration of lead in the muscle of ungulates from mining areas is relatively low (0.08-2.6 µg/g; Taggart et al., 2011; Pareja-Carrera et al., 2014), whereas a single piece of ammunition from a wounded animal can weigh more than 1 mg (Nadjafzadeh et al., 2015). Consequently, vultures would be continuously incorporating small amounts of lead from the topsoil and exceptionally, large quantities from ammunition.

This idea is reinforced by modeling procedures that showed that high levels of blood lead concentrations were related to exposure to ammunition lead. It is well known that ammunition is an agent of clinical lead intoxications in birds of prey (García-Fernández, 2014; Naidoo et al., 2017; Garbett et al., 2018; Krone et al., 2018). More recently, the presence of lead from topsoil and ammunition in griffon vultures has been described (Mateo-Tomás et al., 2016). Nevertheless, to our knowledge, this is the first time the relative contribution of both sources has been studied by integrating stable isotope analysis with fine-scale GPS monitoring.

It could be argued that hunting intensity and topsoil lead exposure show high spatial covariance. These results could be obscuring an additive effect between topsoil and ammunition and can explain the striking differences found in lead concentrations between the two populations, which confirms the findings of Mateo-Tomás et al., (2016) in another region in Spain. Thus, the southern population would be more exposed not only to ammunition (Figure 1) but also to lead in the topsoil. In fact, tissues from wild and domestic ungulates from our southern study area showed high concentrations of lead in contrast to the levels found in these species in other Spanish areas not affected by mining pollution (Santiago et al., 1998; Taggart et al., 2011; Pareja-Carrera et al., 2014). For example, red deer and wild boar from southern study area have lead in muscle of 0.48 and 2.63 respectively. This contrasts with the levels found in these species in other areas not affected by lead mining pollution, where red deer and wild boar showed 0.12 (0.08-0.19) and 0.32 (0.12-0.80) µg/g d.w. of lead in muscle, respectively (Taggart et al., 2011). Similarly, lead concentrations in liver of red deer and wild boar from the mining sites were higher in the southern area (0.43 and 1.92 µg/g) than in control sites (0.11 and 0.39 µg/g) These differences are also noticeable in domestic ungulates. Sheep southern area showed lead levels in liver and muscle of 6.16 (4.12-9.23) and 0.08 (0.07-0.09) µg/g d.w., respectively, which are well above the levels found in sheep from control sites of 0.21 (0.13-0.35) and 0.04 (0.03-0.05) µg/g d.w., in liver and muscle, respectively (Pareja-Carrera et al., 2014). All of this means that griffons feeding on carrion from the southern area can be exposed to lead levels 2 to 8.3-fold greater through a diet of muscle and 4.9 to 29.3-fold higher from liver consumption, which may well partially explain the higher background blood lead concentrations found in griffon vultures from the southern area.

Our models showed that female vultures had higher lead levels that match previous studies in this species (Mateo-Tomás et al., 2016). Our blood samples were taken in winter, coinciding with the beginning of the breeding season and thanks to GPS, we were able to verify that at least 78% of the females and 65% of the males tagged bred during the season in which they were equipped with GPS. Thus, it is reasonable to hypothesize that the sex-based differences could be due to the mobilization of lead from bones occurring during eggshell formation (Gangoso et al., 2009) but certainly further studies would be required to test this hypothesis.

4.2. *Ecological/Physiological Consequences of high lead exposure in vultures*

Almost 80% of the individuals from the southern population and 30% from the northern population were above the threshold value limit established for clinical toxicity (50 µg/dl; Pain et al., 2019). These high lead concentrations are probably related to the fact that the studied vultures were captured in winter, during the big game hunting season (Espín et al., 2014; Hernández & Margalida 2009; Mateo-Tomás et al., 2016; Krone 2018; Garbett et al 2018). In any case, these lead values were above the concentrations described in other species of large avian scavengers (Plaza & Lambertucci 2019; Krüger & Amar 2018) and were comparable to those found in the California condor (*Gymnogyps californianus*) undergoing chelation therapy to counter lead poisoning (Finkelstein et al., 2012). However, we did not detect any deaths attributable to lead intoxication (Arrondo et al., 2020), nor did we perceive intoxication symptoms such as anorexia, dropping head or vomiting in the sampled individuals during the handling process (Krone et al., 2018). This confirms the already described high resistance of griffon vultures to lead exposure (García-Fernandez et al., 2005; Espín et al., 2014). In fact, deaths due to lead exposure are known but seem comparatively rare in relation to other vultures and large body-sized facultative scavenger species (Mateo et al., 1997; Mateo 2009; Horowitz et al., 2014). Beyond the absence of direct mortality and visible symptoms of intoxication, we cannot discard hidden negative effects derived from chronic exposure such as alterations in bone mineralization (Gangoso et al., 2009), physiological effects such as the suppression of δ-ALAD (Espín et al., 2015) or behavioral alterations derived from sub lethal exposures.

5. Further remarks

Topsoil lead can be found naturally (Locutura et al., 2012) but pollution derived from mining activity as occurs in our southern study area is a major problem for wildlife and ecosystems, largely because lead mining activity in Europe has been occurring for millennia (Reglero et al., 2009, Taggart et al., 2011). Although for our target species, no consequences were detected, it is possible to hypothesize that other sensitive threatened species such as Egyptian vultures (*Neophron percnopterus*), red kites (*Milvus milvus*) or Spanish imperial eagles (*Aquila adalberti*) can be affected if their territories and home ranges include highly contaminated mining areas. Consequently, detailed information on topsoil contamination at the level of the entire Iberian Peninsula is necessary to be able to predict damage to wildlife, livestock and human health.

Our results also reinforce the idea that ammunition is the main cause of toxic lead concentration in scavenger birds, such as vultures (García-Fernández 2014; Krone et al., 2018; Pain et al., 2019). This finding is especially relevant in the current context of rural abandonment in which wild ungulates are spreading across Europe as part of a passive rewilding process (Apollonio et al., 2010). In parallel to the growth of wild ungulates populations, hunting pressure is also increasing (Herruzo & Martínez-Jauregui 2013). This inevitably entails a greater exposure to lead and more risk of intoxication for vultures and other scavenger species that consume both the discarded remains of killed animals and the carcasses of mortally injured animals not collected by hunters. In addition, based on our results, exposure to ammunition could be occurring hundreds of kilometers away from the breeding colonies. This is especially relevant for large body-sized scavenging species, which can fly long distances daily crossing administrative boundaries that expose them to different, and sometimes contradictory, legislation (Arrondo et al., 2018). Therefore, the decision to ban lead ammunition partially or at the local scale (Avery & Watson 2009; Mateo & Kanstrup 2019) may be insufficient. It is obvious that a change in legislation regarding the replacement of lead with other materials requires European regulations to develop integral conservation strategies (Lambertucci et al., 2014; Arrondo et al., 2018). This might also contribute to promoting hunting as a more sustainable activity within a rewilding Europe (Kanstrup et al., 2018).

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Table 1: Number and percentage of individuals from the two study areas in each of the categories of lead exposure defined by Pain et al. (2019).

Table 2: Results of the Generalized Linear Models (Gaussian family) performed to determine sources of blood lead concentration in GPS-tagged vultures.

Table S.1: Individual characteristics of the birds included in this study. In column Sex are represented males (M) and females (F). Areas of KDE50 and KDE95 are expressed in km^2. Column Alive indicates if the animals were alive at the end of the study period (December 2018). NA represents those birds whose GPS device failed.

Table S.2: Ammunition and topsoil (control and contaminated) samples used to determine proportion of both lead sources in vultures blood.

Table S.3: Mean and range values of %RSD for replicate analyses (n=6) of vulture blood, topsoil, and ammunition samples.

Table S.4: Statistical summary of the layers used to estimate ammunition and topsoil lead exposure.

Table S.5: AIC-based model selection to assess the lead concentration in vultures. Only models with informative variables are included.

Figure 1: Upper and lower panels show the KDE95 used by all the individuals from northern (blue contour) and southern populations (red contour). Left maps represent the lead concentration in the superficial topsoil (according to Locutura et al., 2012). Right panels show the number of animals hunted per year in 10x10 km^2 cells including the two species most commonly hunted, wild boar and red deer (see methods). Black stars show trapping sites.

Figure 2: Lead isotope ratios (A: $^{207}Pb/^{208}Pb$ - $^{206}Pb/^{207}Pb$; B: $^{206}Pb/^{208}Pb$ - $^{206}Pb/^{207}Pb$; C: $^{206}Pb/^{208}Pb$ - $^{207}Pb/^{208}Pb$) in blood of griffon vultures from northern and southern populations. Red and blue dotes represent southern and northern population individuals, respectively. Mean and standard deviation of lead isotope ratios of the two lead sources (ammunition and topsoil) are also shown.

Figure 3: Mean and 95% Confidence Interval of the estimated contribution of lead from ammunition and topsoil to blood lead concentration in vultures from both northern and southern populations, based on the results of the MixSIAR models.

Table 1

		Blood Pb concentration (µg/dl) N(%)			
Population	Sex	<20 Background	20-50 Sublethal effects	50-100 Clinical effects	>100 Potentially lethal
Northern	Female	2(12.5)	8(50.0)	5(31.3)	1(6.3)
	Male	4(28.6)	6(42.9)	2(14.3)	0(0.0)
	Total	6(21.4)	14(50.0)	7(25.0)	1(3.6)
Southern	Female	0(0.0)	3(27.3)	5(45.5)	3(27.3)
	Male	2(10.5)	5(26.3)	11(57.9)	1(5.3)
	Total	2(6.7)	8(26.7)	16(53.3)	4(13.3)
Both	Female	2(7.4)	11(40.7)	10(37.0)	4(14.8)
	Male	6(18.2)	11(33.3)	13(39.4)	1(3.0)
	Total	8(13.8)	22(37.9)	23(39.7)	5(8.6)

Table 2

Variables	Estimate±Std. Error	p-value
(Intercept)	3.438±0.159	<0.001
exposure to ammunition from big game hunting at KDE 50	0.004±0.001	<0.001
males	-0.337±0.153	0.003
(Intercept)	3.438±0.167	<0.001
exposure to ammunition from big game hunting at KDE 95	0.004±0.001	<0.001
males	-0.327±0.155	0.004
(Intercept)	3.141±0.196	<0.001
exposure to topsoil lead at KDE95	0.022±0.006	<0.001

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Table S.1.

Individual	Alive	Population	Sex	KDE50	KDE95
L73	YES	Southern	M	70.88	1124.7916
L8J	YES	Southern	M	243.41	4668.9676
T00	YES	Southern	F	207.61	3426.2532
T01	YES	Southern	F	318.63	5675.5555
T02	YES	Southern	M	172.82	2492.7401
T03	YES	Southern	M	519.83	6090.678
T05	YES	Southern	M	78.10	5457.2433
T06	NA	Southern	M	84.64	2768.1997
T07	NO	Southern	M	59.07	2666.8988
T08	YES	Southern	F	196.74	3326.8942
T09	YES	Southern	M	81.93	4137.2157
T0A	YES	Southern	M	334.30	2798.9276
T0C	YES	Southern	M	105.33	4678.9345
T0H	YES	Southern	M	307.49	3596.8582
T0J	YES	Southern	F	964.21	8335.799
T0L	NA	Southern	M	55.26	3110.0696
T0U	NO	Southern	F	187.80	3960.7735
T0V	YES	Southern	M	231.85	2922.7373
T0W	NA	Southern	F	57.60	2701.8798
T0X	NA	Southern	M	164.98	5371.7382
T10	NA	Southern	F	464.65	5438.5837
T11	YES	Southern	M	51.72	5323.4727

T12	NA	Southern	M	83.62	2455.8705
T14	YES	Southern	M	226.10	4117.9899
T15	YES	Southern	F	780.49	8514.226
T16	YES	Southern	M	92.43	1898.6661
T17	YES	Southern	M	247.02	7765.4193
T19	NO	Southern	F	309.69	3856.2093
T1C	NA	Southern	F	462.86	3382.1542
T1J	YES	Southern	F	780.26	7626.0972
T1L	YES	Northern	M	57.71	1336.3249
T1N	YES	Northern	F	86.82	871.3995
T1R	YES	Northern	M	205.56	2111.6737
T1U	NO	Northern	F	134.95	891.8856
T1W	NO	Northern	M	46.04	1760.9609
T1X	YES	Northern	F	209.66	2653.92
T21	YES	Northern	M	38.42	543.4831
T22	NO	Northern	M	25.55	233.7521
T24	YES	Northern	F	88.19	1029.8596
T25	YES	Northern	M	698.48	7775.0831
T2C	NO	Northern	M	88.75	995.9196
T2F	YES	Northern	F	73.39	791.1746
T2H	YES	Northern	M	79.03	678.0347
T2L	YES	Northern	F	191.61	3106.063
T2M	YES	Northern	M	142.04	1890.6208
T2N	NO	Northern	M	34.25	536.0871
T2R	YES	Northern	F	738.00	7057.0544
T2T	YES	Northern	F	128.09	908.3614
T2U	YES	Northern	M	210.53	2475.1838
T2V	YES	Northern	F	152.63	2338.3821
T2W	YES	Northern	F	302.94	4039.008

T2X	NO	Northern	F	54.22	1311.9559
T30	YES	Northern	F	66.13	1967.6696
T31	YES	Northern	F	362.49	5181.7917
T33	YES	Northern	F	271.52	2665.2463
T35	NO	Northern	F	132.29	2478.3662
T36	YES	Northern	M	20.05	142.9229
T3T	NO	Northern	F	71.20	518.6238

Table S.2.

ID	N	Kind of lead	206/207	207/208	206/208
Topsoil T05_10 A	1	Control topsoil	1.1934	0.4004	0.4778
Topsoil T05_10 B	1	Control topsoil	1.1945	0.3996	0.4773
Topsoil T05_10 C	1	Control topsoil	1.1711	0.4059	0.4754
Topsoil T05_9	1	Control topsoil	1.1931	0.4015	0.4790
Topsoil NM2	1	Contaminated topsoil	1.1711	0.4073	0.4770
Topsoil Pto 122 B	1	Contaminated topsoil	1.1626	0.4106	0.4774
Topsoil Pto 123	1	Contaminated topsoil	1.1580	0.4097	0.4744
Topsoil Pto 124	1	Contaminated topsoil	1.1536	0.4112	0.4743
Topsoil Pto 125	1	Contaminated topsoil	1.1542	0.4098	0.4730
Topsoil Pto 133	1	Contaminated topsoil	1.1549	0.4103	0.4738
Topsoil Pto 74	1	Contaminated topsoil	1.1526	0.4119	0.4748
Norma 3006 A	3	Bullet	1.1455	0.4141	0.4743
Norma 3006 B	3	Bullet	1.1544	0.4130	0.4768
S&B 3006	2	Bullet	1.1473	0.4118	0.4724

REMINGTON 270	1	Bullet	1.2323	0.4051	0.4992
REMINGTON 300 A	2	Bullet	1.2241	0.4058	0.4968
REMINGTON 300 B	3	Bullet	1.2003	0.4087	0.4905
WINCHESTER 270 WSM	2	Bullet	1.2000	0.4091	0.4910
HORNADY 300	2	Bullet	1.2018	0.4094	0.4920
BROWING 12	3	Cartridge	1.1529	0.4132	0.4764

Table S.3

Sample	206/207	207/208	206/208
Vulture blood	0.124 (0.029-0.229)	0.147 (0.035-0.279)	0.140 (0.004-0.271)
Topsoil	0.140 (0.080-0.245)	0.134 (0.049-0.231)	0.127 (0.057-0.205)
Ammunition	0.138 (0.097-0.183)	0.138 (0.064-0.277)	0.119 (0.067-0.193)

Table S.4

Variables	Minimum	Maximum	Mean	Standard Deviation
Hunting exposure	0	320	70.07	59.90
Topsoil lead exposure	1	8545	27.22	54.32

Table S.5

Model	AICc	ΔAICc	Cumulative weight	Weight	R^2
exposure to ammunition from big game hunting at KDE 50+sex	**104.77**	**0**	**0.44**	**0.44**	**23.8**
exposure to ammunition from big game hunting at KDE 95+sex	**106.44**	**1.67**	**0.19**	**0.63**	**21.9**
exposure to topsoil lead at KDE95	**106.63**	**1.86**	**0.17**	**0.8**	**18.1**
exposure to ammunition from big game hunting at KDE 50	107.36	2.59	0.12	0.92	
exposure to ammunition from big game hunting at KDE 95+sex	108.66	3.89	0.06	0.98	
exposure to topsoil lead at KDE50	111.53	6.76	0.01	1	
NULL	116.02	11.25	0	1	

Figure 1

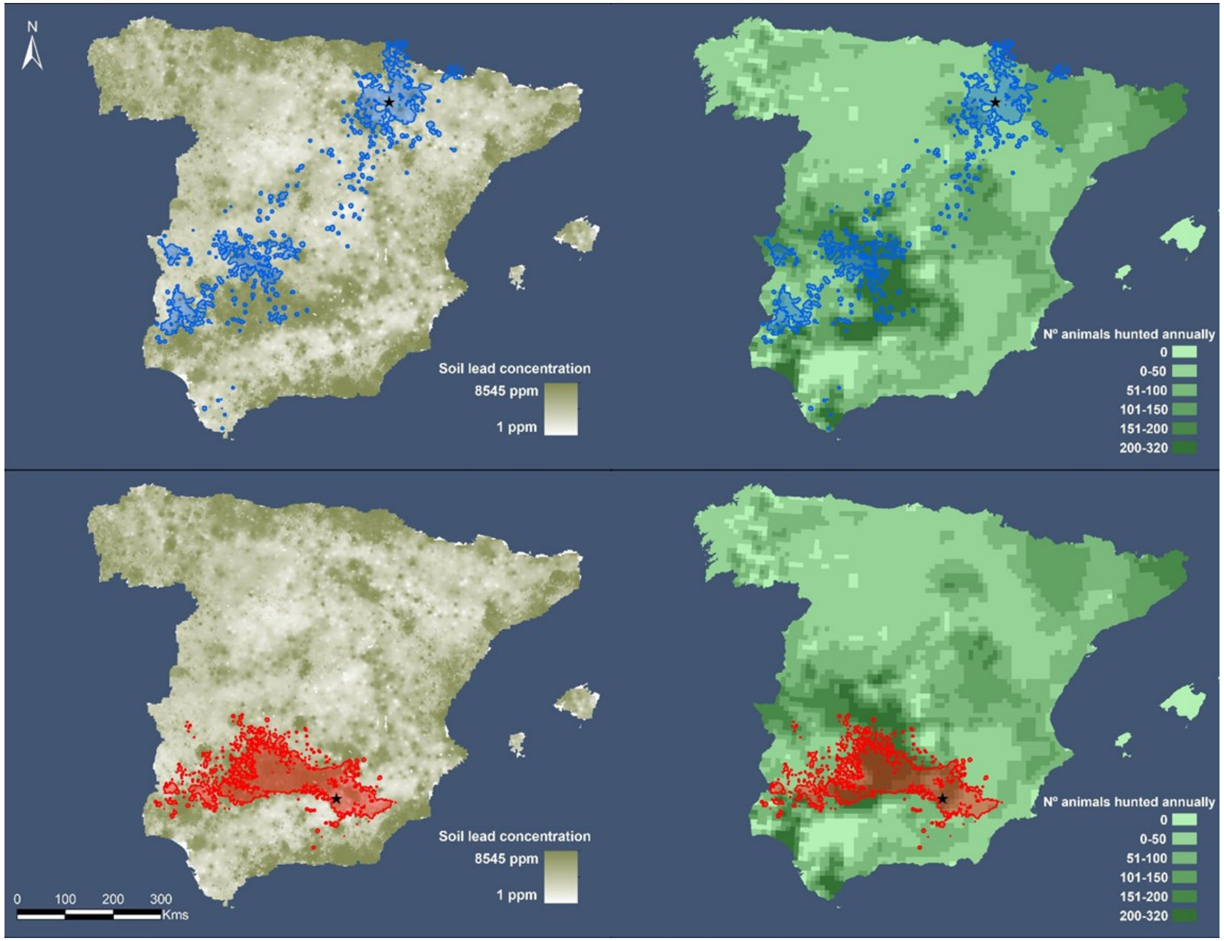

N
Soil lead concentration
8545 ppm
1 ppm
Nº animals hunted annually
0
0-50
51-100
101-150
151-200
200-320
Soil lead concentration
8545 ppm
1 ppm
0
100
200
300
Kms
Nº animals hunted annually
0
0-50
51-100
101-150
151-200
200-320

Figure 2

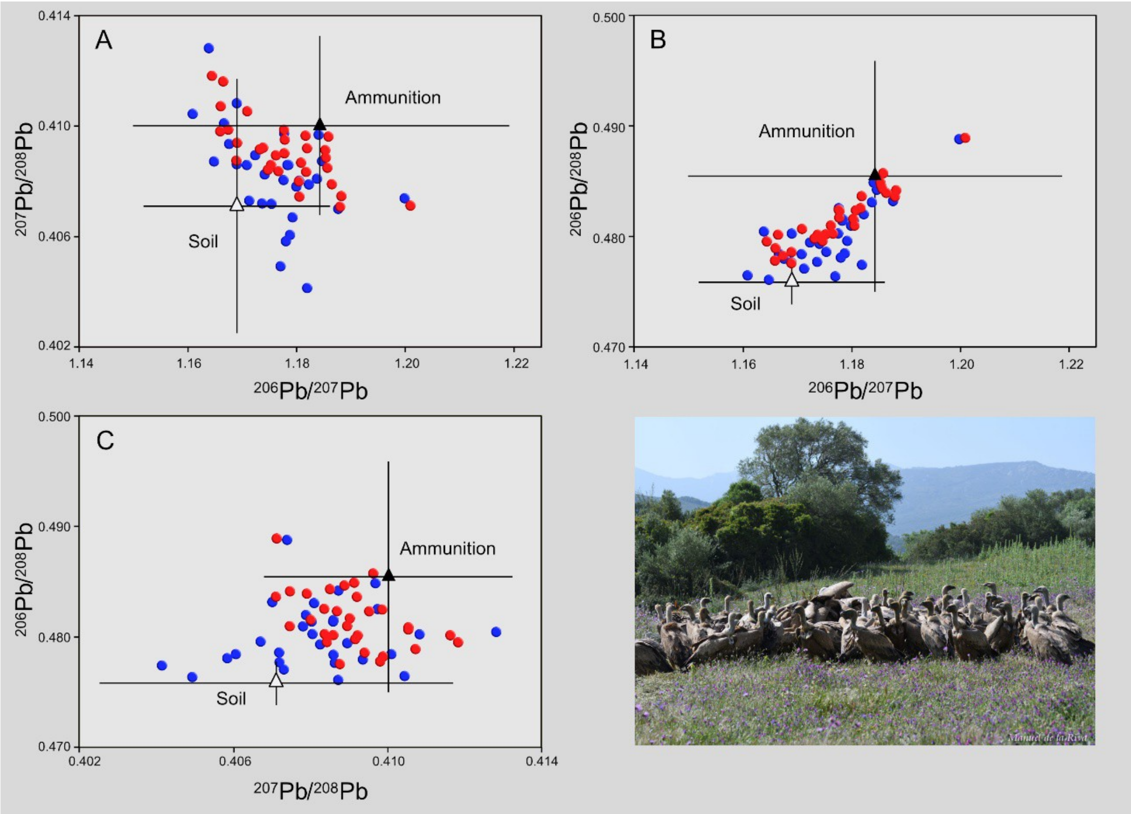
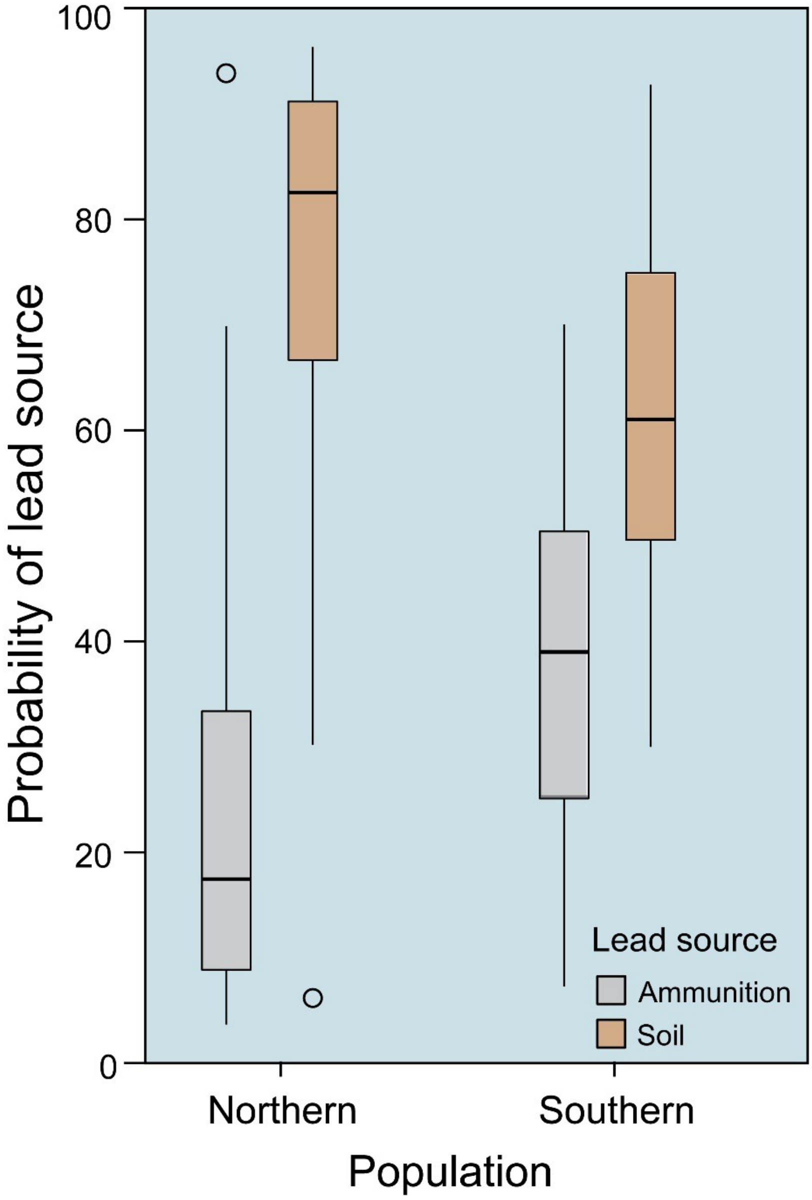

A
Ammunition
Soil
^{207}Pb/^{208}Pb
^{206}Pb/^{207}Pb
B
Ammunition
Soil
^{206}Pb/^{208}Pb
^{206}Pb/^{207}Pb
C
Ammunition
Soil
^{206}Pb/^{208}Pb
^{207}Pb/^{208}Pb

Figure 3


100
80
60
40
20
0
Probability of lead source
Northern
Southern
Population
Lead source
Ammunition
Soil